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(54) Title: TARGETED PROTECTION FROM CYTOTOXINS

(57) Abstract

A method of targeted rescue of cells from the effect of a chemotherapeutic cytotoxin is disclosed. A cytotoxin directed against diseased cells is administered with an antagonist-conjugate targetable to normal cells. The antagonist-conjugate is made up of an antagonist of the cytotoxin complexed with a cell-specific binding agent which specifically binds to a cellular surface component present on normal, but not diseased cells. The cellular surface component is typically a surface receptor of the cell which mediates endocytosis. The antagonist-conjugate is specifically taken up by normal cells to provide protection from the toxic effects of the cytotoxin. Diseased cells which lack the surface receptor do not take up effective amounts of the antagonist and consequently are unprotected.

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TARGETED PROTECTION FROM CYTOTOXINS

Background of the Invention

Chemotherapeutic agents currently available for treatment of tumors can be unsuccessful because they lack tumor specificity. The use of galactosamine has been explored in the treatment of primary liver cancer (hepatocellular carcinoma) because it is a highly selective liver toxin in vitro and in vivo. The selectivity is due to elevated intrahepatic levels of two enzymes of the galactose metabolic pathway, galactokinase, and UDP-glucose:galactose-1-P-uridyltransferase (Bertoli, D. and Segal, S. (1966) J. Biol. Chem. 241:4023 and Cuatrecasas, P. and Segal, S. (1965) J. Biol. Chem. 240:2382), that allow galactosamine to be metabolized as a galactose analog (Keppler, D. and Decker K., (1969) Eur. J. Biochem. 10:219). This eventually leads to trapping and depletion of intracellular uridine intermediates in hepatocytes and hepatocyte-derived cells (Keppler, D.O.R., et al. (1970) Eur. J. Biochem. 17:246). However, high doses of galactosamine sufficient to destroy hepatoma cells results in toxicity to normal hepatocytes. It has been shown that a galactosamine antagonist can be targeted to hepatocytes, specifically protecting them from galactosamine toxicity in vitro (Wu, G.Y., et al. (1988) J. Biol. Chem. 263: 4719).

A method of protecting normal cells in vivo from the cytotoxic activity of the chemotherapeutic agents may alleviate the problems of toxicity and enhance the effectiveness of these agents.

Summary of the Invention

This invention pertains to a method of selectively protecting normal cells from the cytotoxic effects of a chemotherapeutic cytotoxin directed against diseased cells such as tumor cells. According to the method, the chemotherapeutic cytotoxin is administered in conjunction with, or subsequent to, administration of an antagonist-conjugate. The antagonist-conjugate comprises an antagonist of the cytotoxin coupled to a cell-specific binding agent which binds to a cellular surface component present on normal, but not on diseased cells. The cellular surface component is typically a receptor which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The antagonist can be complexed with the cell-specific binding agent via an antagonist-binding agent, such as a polycation.

The antagonist-conjugate is administered in vivo where it is selectively taken up by normal cells via the surface-structure-mediated endocytotic pathway. The conjugate is administered in an amount sufficient

to protect normal cells from the cytotoxic effects of the cytotoxin. Diseased cells which lack the cellular surface component do not take up significant amounts of the antagonist-conjugate and are unprotected from the cytotoxin. The method provides for a more effective use of higher doses of cytotoxins against tumor and against other diseases by alleviating or eliminating the toxicity to normal cells usually associated with such therapy.

Brief Description of the Figures

Figure 1 shows the organ distribution of radiolabeled galactosamine antagonist-conjugate.

Figure 2 shows the effect of galactosamine antagonist-conjugate pretreatment on galactosamine toxicity.

Detailed Description of the Invention

This invention pertains to a method of selectively targeting an antagonist of a cytotoxin to normal mammalian cells to protect against the adverse effects of a therapeutic cytotoxin. An antagonist-conjugate targetable to normal mammalian cells is used to selectively deliver an antagonist to the cells in vivo. The antagonist-conjugate comprises an antagonist of the cytotoxin complexed with a cell-specific binding agent which binds a cellular surface component present on normal, but not diseased cells. The antagonist-conjugate is selectively taken up by the normal mammalian cells and the antagonist is released into the cell in functional form to provide protection against the effects of the cytotoxin.

The cell-specific binding agent specifically binds a cellular surface component which mediates internalization by, for example, the process of endocytosis. The surface component can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the surface component can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The cell-specific binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody which binds the cellular surface component.

Ligands useful in forming the antagonist-conjugate will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands

can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive amination.

For targeting the antagonist-conjugate to other cellular surface components, other types of ligands can be used, such as mannose for macrophages, mannose-6-phosphate glycoproteins for fibroblasts, intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specifc binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The antagonist-conjugate can be made by binding the antagonist directly to the ligand or by binding it with the ligand through an antagonist-binding agent. The antagonist-binding agent complexes the antagonist to be delivered. Complexation with the antagonist must be sufficiently stable in vivo to prevent significant uncoupling of the antagonist extracellularly prior to internalization by the cell. However, the complex is cleavable under appropriate conditions within the cell so that the antagonist is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic attraction between the antagonist-binding agent and the antagonist provides extracellular stability and is releasable under intracellular conditions.

Preferred antagonist-binding agents are polycations which provide multiple binding sites for antagonists. Examples of polycations include polylysine, polyornithine or histones.

The antagonist-binding component can be covalently bonded to the ligand. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G., et al. (1981) Biochem. Biophys. Res. Commun. 101:599-606. An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular antagonist-binding agent and ligand used to form the conjugate. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the conjugate components. The optimal ratio of antagonist-binding agent to ligand can be determined empirically.

Uncoupled components and aggregates can be separated from the conjugate by molecular sieve chromatography.

The conjugate can contain more than one antagonist molecule or one or more different antagonist molecules. Preferably, from about 10-15 antagonist molecules per conjugate. The number may vary, depending upon factors such as the effect on solubility or capillary permeability of the conjugate.

The cytotoxin and antagonist can be selected from any of those effective in treatment of the disease. For tumor therapy, various antitumor agents for which antagonists are available can be used. Examples of antitumor cytotoxins and corresponding antagonists include methotrexate/folinic acid, acetaminophen/ N-acetyl cysteine, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)/N-acetyl cysteine, glutathione or WR2721 and galactosamine/uridine monophosphate or orotic acid. In addition, combinations of two

different cytotoxins and respective antagonists (which may be the same or different) can be used to reduce selection of resistant cells.

In a preferred embodiment, the cytotoxin is specific for the diseased organ or tissue. This helps minimize toxicity of uninvolved organs. For example, as described, galactosamine is a highly selective hepatotoxin and therefore, is preferred for treatment of primary liver cancer such as hepatocellular carcinoma.

In preferred embodiments, the antagonist-conjugate is soluble in physiological fluids. The antagonist-conjugate is generally administered parenterally in a physiologically acceptable vehicle in an amount sufficient to protect normal cells against the toxic effects of a cytotoxin.

The invention is illustrated further by the following exemplification.

Exemplification

Preparation of an AsF-PL-UMP Conjugate

The asialoglycoprotein, asialofetuin (AsF) was prepared by desialylation of bovine fetuin (GIBCO, Grand Island, New York), using neuraminidase (Sigma Chemical Co., St. Louis, Missouri) to expose terminal galactose residues by a modification of the method of Oka and Weigel (Oka, J.A. and Weigel P.H. (1983) J. Biol. Chem. 258:10253). Analysis of residual protein-bound sialic acid by the method of Warren (Warren, L. (1959) J. Biol. Chem. 234:1971) determined the fetuin to be 94% desialylated.

In order to create a targetable carrier protein with a large capacity to bind antagonist, AsF, 55 μM was coupled to poly-L-lysine (PL) (Sigma Chemical Co., St. Louis, Missouri) 470 μM, Mr = 3600 using 1-ethyl-3-(3-dimethylamino)propyl carbodiimide (Pierce Chemical Co., Rockville, IL) as described previously (Wu, G.Y., et al. (1988) J. Biol. Chem. 263:4719). 5'-Uridine monophosphate (UMP) (Sigma Chemical Co., St. Louis, Missouri) was then coupled to the carrier protein according to the method of Halloran and Parker (Halloran, M.J. and Parker, C.W. (1966) J. Immunol. 96:373) purified by column chromatography (Wu, G.Y., et al. (1988) J. Biol. Chem. 263:4719). The conjugate was stable at 4°C for at least two weeks.

Organ Distribution of Injected AsF-PL-UMP Conjugate

To determine whether the conjugate retained its ability to be recognized by asialoglycoprotein receptors in vivo both AsF and the AsF-PL-UMP conjugate were radiolabeled with Na ^{125}I (Amersham Corporation, Chicago, Illinois) using a solid-phase lactoperoxidase method as described by the manufacturer (BioRad). Female Sprague-Dawley rats (220-270g) (Zivic-Miller Laboratories, Allison Park, Pennsylvania) were injected intravenously with sterile saline containing 1 μg ^{125}I-AsF, or 1 μg ^{125}I AsF-PL-UMP (based on AsF content). To determine whether liver uptake of the conjugate was via asialoglycoprotein receptors, a control rat was given 1 μg ^{125}I-AsF-PL-UMP plus an excess, 10 mg, of unlabeled asialoorosomucoid (AsOR) to compete for hepatic asialoglycoprotein receptors. To evaluate the extent of non-specific hepatic uptake of conjugate, 15

mg of dextran sulphate (Pharmacia, Upssala, Sweden, Na), an inhibitor of nonparenchymal "scavenger" receptor activity, was administered intravenously, 15 minutes prior to the conjugate injection according to the method of Van der Sluijs, et al. (Van Der Sluijs, P., et al. (1986) Hepatology 6:723). Another control received both dextran sulphate and excess AsOR. Ten minutes after injection of labeled protein, blood was drawn from the retro-orbital plexus and the animals sacrificed. The distribution of radioactivity among organs was determined by gamma counting and expressed as percent of total counts.

As shown in Figure 1, for rats receiving either conjugate or AsF, approximately 80% of the counts were taken up by the liver. The addition of excess AsOR successfully competed with the labeled conjugate for hepatic asialoglycoprotein receptors resulting in removal by liver of only 16% of the injected counts. The inhibition of uptake of ^{125}I-AsF-PL-UMP by competition with the excess AsOR indicates that the targeting of the antagonist was directed by the asialoglycoprotein component of the conjugate. The lack of effect of UMP injected alone in identical amounts and under identical conditions as for the conjugate argues against intravascular cleavage of UMP from the conjugate as a mechanism of the observed protection by the conjugate.

Injection of dextran sulphate, which inhibits non-specific uptake via nonparenchymal "scavenger" receptors, had no effect on liver uptake of the conjugate which still accounted for 80% of the injected radioactivity. Administration of both dextran sulphate and excess AsOR had no further effect

on liver uptake of the conjugate beyond that of excess AsOR alone. These data indicate that neither the PL, UMP nor the process of conjugation had altered recognition of the AsF by hepatic asialoglycoprotein receptors in the intact rat.

Potential toxicity of the conjugate itself was evaluated by injecting conjugate alone at the dose and volume used in the previous experiment (34 mg/kg). The animal was observed at various time intervals, then sacrificed at 42 h (the time of peak galactosamine toxicity, and therefore of maximal conjugate protection in the previous experiment). Various organs and tissues were removed and prepared for histological examination.

The behavior of a rat receiving conjugate alone showed no obvious indications of cardiopulmonary distress or neurological deficits. No abnormalities were revealed by histological examination of liver, kidney, spleen, heart and surrounding large vessels, lungs and trachea, brain, peripheral nerves and ganglia, adrenal gland, lymph nodes, skeletal muscle and adipose tissue.

Effect of AsF-PL-UMP Conjugate on Galactosamine Toxicity

As shown in Figure 2, the effect of the targeted antagonist on galactosamine toxicity to hepatocytes in vivo was determined. To allow sufficient time for internalization of the conjugate and release of the antagonist, the targetable antagonist-conjugate was injected i.v. (in 5 ml sterile saline) in female rats as a 2 h pretreatment prior to the galactosamine injection. Subsequently, rats were injected

intraperitoneally with 800 mg/kg galactosamine (Sigma Chemical Co., St. Louis, Missouri), in 2.5 ml sterile saline, pH 7.4. The minimum amount of conjugate required to protect hepatocytes was determined by i.v. injection of varying doses of conjugate. Using the conjugate dose thus determined optimal (34 mg/kg), the ability of this antagonist conjugate to prevent galactosamine toxicity was evaluated relative to controls receiving i.v. injected pretreatments of equal volumes of sterile saline, or saline containing AsF or UMP in molar amounts equivalent to that provided by the conjugate. Blood was withdrawn from the retro-orbital plexus at 24, 42, 48 and 72 h after galactosamine injection. Hepatotoxicity was evaluated by measurement of serum alanine aminotransferase (ALT) levels (Sigma assay kit) according to the manufacturer. All assays were performed in duplicate and expressed as international units per liter (IU/l). Addition of conjugate to ALT standards as well as serum samples demonstrated that the conjugate had no effect on the ALT assays.

A Kruskal-Wallis test was used to evaluate differences among the four groups (6-7 rats per treatment; pretreatment ALT values averaged 38 with S.D. of 9). Once a significant difference among treatments was determined, pair-wise comparisons were evaluated with Wilcoxon-Mann-Whitney tests (Zar J.H., 1984, Biostatistical analysis. Prentice-Hall, Englewood Cliffs, N.J.).

Selective uptake by the liver of conjugate in trace amounts, demonstrated above, was also found for this higher dose of conjugate. Because ALT values were compared at this peak 42 h (always decreasing by 72 h). Serum ALT values were compared at this peak 42 h time point. A Kruskal-Wallis test determined that there were significant differences among the four groups, with an alpha level of 0.01. Pair-wise comparisons (Wilcoxon-Mann-Whitney tests) determined that animals pretreated with AsF-PL-UMP conjugate experienced significantly less hepatotoxicity than saline controls as measured by serum ALT levels ($p<.005$). Animals that received conjugate likewise had significantly lower ALT values than those receiving either AsF alone or UMP alone ($p<.05$ and $p<.002$ respectively). There were no significant differences among the three controls.

The results indicate that the AsF-PL-UMP conjugate can be targeted to hepatocytes resulting in protection of these cells from galactosamine toxicity in vivo. The lack of effect of administration of UMP alone can be explained by the fact that uridine in the form of the conjugate was targeted only to hepatocytes while free UMP could be dispersed by the circulation for uptake throughout the body. Unlike the UMP in the form of the conjugate, free UMP provided to the liver from the circulation was evidently inadequate to prevent galactosamine toxicity.

AsOR-PL-UMP Conjugate

An AsOR-PL-UMP conjugate was produced by the method as described above. Using this conjugate, the effect on galactosamine toxicity was determined. Improvement in protection as compared to the AsF-PL-UMP conjugate was achieved by i.v. infusion of the AsOR-PL-UMP conjugate over a 4 h period (at the saturation rate of hepatic asialoglycoprotein receptors). At a galactosamine dose of 500 mg/kg, median peak alanine aminotransferase (ALT) levels for AsOR-PL-UMP infused rats were 1725, compared to 3059 for saline infused controls.

AsOR-Taurine Conjugate

To achieve better protection of normal liver from high doses of galactosamine, different antidote conjugates were developed. The final irreversible step in galactosamine toxicity is an influx of Ca^{++} into the damaged cells. Since taurine can interfere with toxicity by causing intracellular sequestration of calcium, an AsOR-taurine conjugate was developed as described above. Administration of the AsOR-taurine conjugate after galactosamine provided no protection. When the conjugate was infused over a 4 h period prior to administration of 500 mg/kg galactosamine, it provided protection (median peak ALT 820 compared to 2282 in saline-infused controls). Protection of conjugate-pretreated animals was increased by administering uridine (1.2 g/kg) 5 h after galactosamine (a point when galactosamine damage in saline controls should be irreversible). With this strategy, conjugate (+ uridine) treated animals experienced median peak ALT levels of 258, compared to 729 for saline (+ uridine) treated controls.

AsOR-PL-Orotic Acid Conjugate

Following the procedure as described for the AsF-PL-UMP conjugate, another targetable antidote conjugate was developed containing the UMP precursor, orotic acid. Administration of this AsOR-PL-orotic acid conjugate resulted in superior protection of normal hepatocytes in vivo from higher doses of galactosamine. The addition of an agent that blocks de novo synthesis of uridylates, such as N-phophonacetyl 6-aspartate (PALA), with galactosamine should increase toxicity to hepatomas. Pretreatment with the orotic acid conjugate (infused over 4 h) provided significant protection from PALA plus even higher doses of galactosamine (Table 1).

Table I: Effect of a Targetable Orotic Acid Conjugate on Hepatotoxicity and Survival in Rats Treated with Hepatotoxins

Hepatotoxin	Dose (mg/kg)	Pretreatment	Median Serum ALT Levels	Percent Survival
Galactosamine	500	Carrier-orotic acid conjugate	503	100
		Saline alone	3,329	100
		Carrier alone	2,883	100
Galactosamine +PALA (N-Phosphonacetyl L-Aspartate)	800;20	Carrier-orotic acid conjugate	3,012	100
		Saline alone	>10,000	0
		Carrier alone	8,382	0
		Orotic acid alone	>10,000	0

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method of treating a disease, comprising administering to an individual afflicted with the disease a cytotoxic amount of a cytotoxin directed against diseased cells and an antagonist-conjugate targetable to normal cells in an amount sufficient to protect the normal cells against the cytotoxic effect of the cytotoxin, the antagonist-conjugate comprising an antagonist of the cytotoxin complexed with a cell-specific binding agent which specifically binds a cellular surface component present on normal, but not diseased cells.

2. A method of claim 1, wherein the disease is a tumor.

3. A method of claim 2, wherein the tumor is a hepatic tumor.

4. A method of claim 3, wherein the hepatic tumor is hepatic carcinoma.

5. A method of claim 1, wherein the cytotoxin is organ-specific.

6. A method of claim 5, wherein the cytotoxin is galactosamine and the antagonist is selected from the group consisting of uridine monophosphate, taurine, or orotic acid.

7. A method of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

8. A method of claim 7, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

9. A method of claim 8, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

10. A method of claim 1, wherein the antagonist is coupled to the cell-specific binding agent via an antagonist-binding agent.

11. A method of claim 10, wherein the antagonist-binding agent is a polycation.

12. A method of claim 11, wherein the polycation is polylysine.

13. A method of claim 1, wherein a combination of at least two different cytotoxins are administered.

14. A method of claim 1, wherein the antagonist-conjugate is administered prior to administration of the cytotoxin.

15. A method of treating a hepatic tumor, comprising administering to a person afflicted with the tumor, an anti-tumor amount of hepatotoxin and an antagonist-conjugate targetable to normal cells in an amount sufficient to protect normal hepatocytes from the toxic activity of the hepatotoxin, the antagonist-conjugate comprising an antagonist of the hepatotoxin complexed with a ligand for the asialoglycoprotein receptor present on normal, but not the tumorous, hepatocytes.

16. A method of claim 15, wherein the hepatic tumor is hepatocellular carcinoma.

17. A method of claim 15, wherein the hepatotoxin is galactosamine, the antagonist is selected from the group consisting of uridine monophosphate, taurine or orotic acid, and the ligand is an asialoglycoprotein.

18. A method of claim 15, wherein the antagonist is complexed with the ligand via a polycation.

19. A method of claim 18, wherein the polycation is polylysine.

20. A method of claim 15, wherein the antagonist-conjugate is administered prior to administration of the hepatotoxin.

21. A method of claim 20, wherein the antagonist-conjugate is administered at a rate that saturates the hepatic asialoglycoprotein receptors.

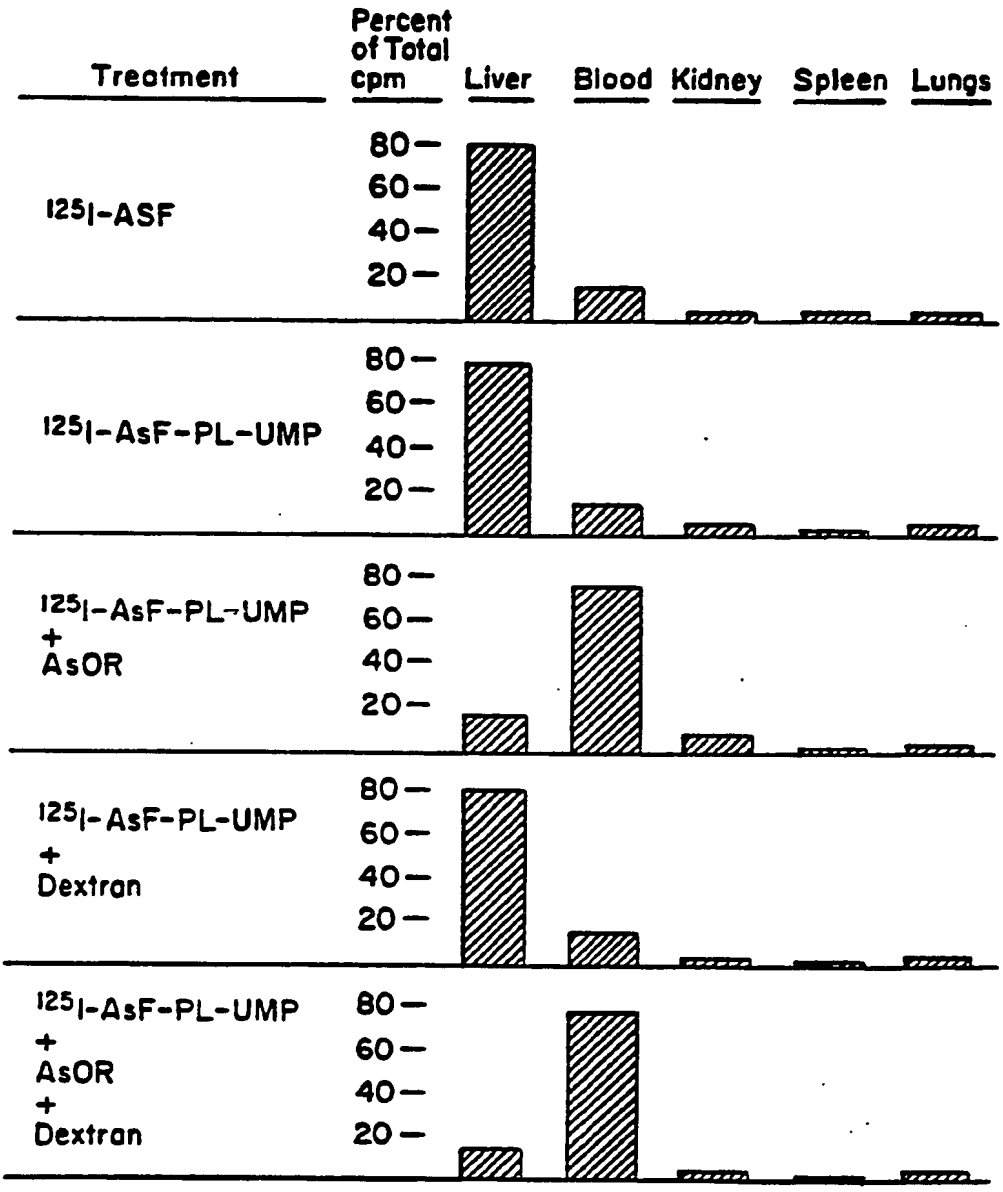
Treatment
Percent of Total cpm
Liver
Blood
Kidney
Spleen
Lungs
125I-ASF
125I-AsF-PL-UMP
125I-AsF-PL-UMP + AsOR
125I-AsF-PL-UMP + Dextran
125I-AsF-PL-UMP + AsOR + Dextran
80
60
40
20

FIG. 1

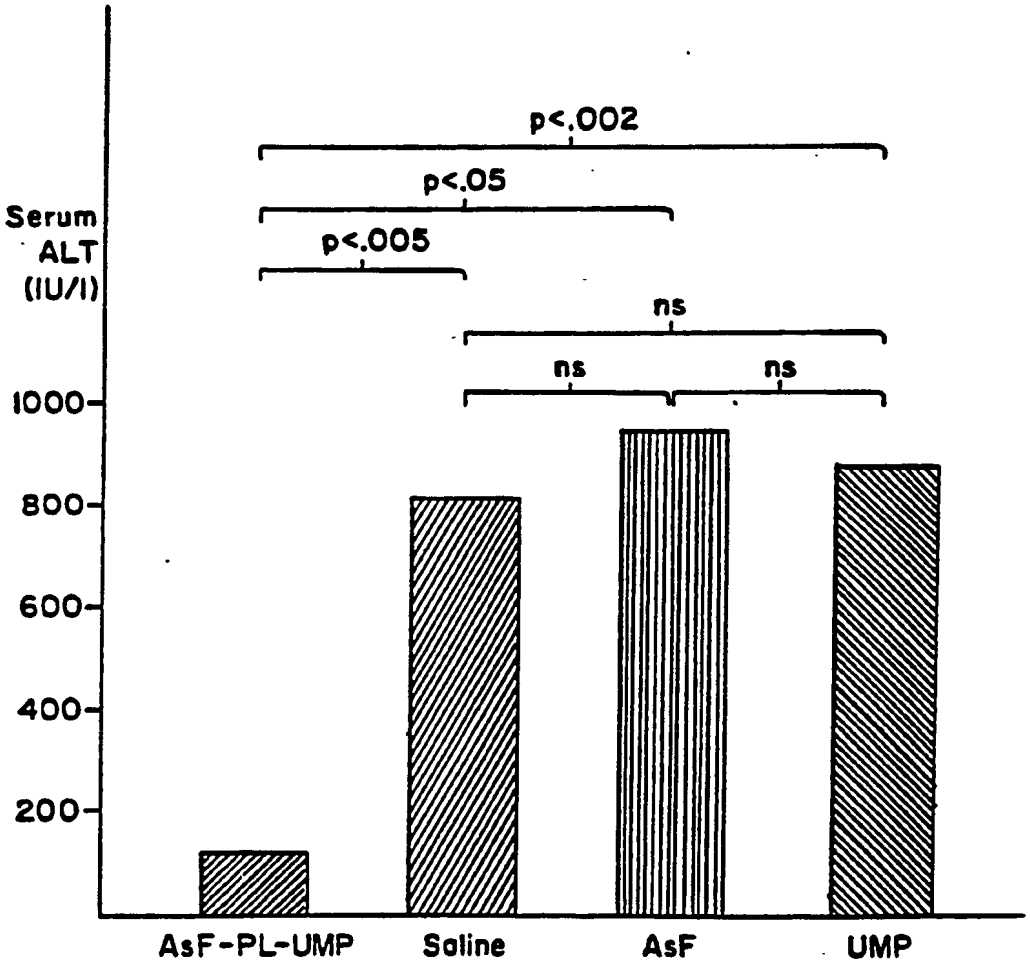
p<.002
p<.05
p<.005
ns
ns
ns
Serum ALT (IU/l)
1000
800
600
400
200
AsF-PL-UMP
Saline
AsF
UMP

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03291

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02, 31/505; C07K 17/02
U.S. CL.: 514/8, 12, 21, 51; 530/395, 409

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/8, 12, 21, 51; 530/395, 408, 409

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS, BIOSIS, MEDLINE COMPUTER files.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Cancer Chemother and Pharmacol., volume 26, issued 1990, KEEGAN-ROGERS ET AL., "Targeted protection of hepatocytes from galactosamine toxicity in vivo", pages 93-96, see entire document.	1-20 21
X Y	The Journal of Biological Chemistry, volume 263, No. 10, issued 05 April 1988, WU ET AL., "Targeted Antagonism of galactosamine toxicity in normal rat hepatocytes in vitro" pages 4719-4723, see entire document.	1-21 1-21
Y	Hepatology, volume 5, No. 5, issued 1985, WU ET AL., "Acetaminophen hepatotoxicity and targeted rescue: A model for specific chemotherapy of hepatocellular carcinoma", pages 709-713, see page 709.	1-21
Y	Proc. Natl. Acad. Sci. USA, Volume 80, issued May 1983, WU ET AL., "Model for specific rescue of normal hepatocytes during methotrexate treatment of hepatic malignancy", pages 3078-3080, see 3078.	1-21

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 August 1991	20 AUG 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Kay. K. Kim, Ph.D. (vsh)

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